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Human immune response to rabies nucleocapsid and glycoprotein antigens

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SUMMARY

Antibodies to two components of rabies virus, nucleocapsid (N) and glycoprotein (G), were compared in 11 rabies patients with those in nine recipients of Vero cell rabies vaccine. All rabies vaccinees had antibodies to N and G components by day 10 after the first vaccine injection. A similar but not identical response was observed in three out of 11 rabies patients. Serum antibodies appeared in rabies patients as early as 3 days after onset of the first symptoms of the disease. In these antibody-positive rabies patients, levels of both antibodies, but particularly of anti-N antibody, were lower than in the vaccinated group. Our results suggest that the process of immune recognition and of antibody development in human rabies is more likely to occur early in the pre-clinical phase, and that reactivity to N protein may be crucial for elicitation of neutralizing antibody.

Keywords human rabies humoral immune response nucleocapsid protein glycoprotein

INTRODUCTION

Rabies is regarded as a virtually inevitably fatal disease in humans. Once disease signs develop, no intervention has been able to prevent death. Rabies virus is usually transmitted by animal bites. Initial replication occurs at the site of inoculation in striated muscle cells (Harrison & Murphy, 1978). Once rabies virus has gained entry to the nervous system, rapid dissemination by direct cell to cell transmission or trans-synaptic spread occurs (Iwasaki & Clark, 1975, Perl 1975, Charlton & Casey, 1979). Absence of viraemia and infection at immunoprivileged sites, such as the nervous system, may have implications for the failure of host defence mechanisms to eradicate an established CNS infection with street virus.

Our recent studies in human rabies have shown that cell-mediated immune response to rabies virus may occur at the time patients exhibit early signs of rabies, and that immune responses may aggravate the severity of the disease (Hemachudha *et al.*, 1988). Patients who have cellular reactivity to rabies virus, as determined by the lymphocyte proliferation test, usually manifest as encephalitis rather than paralysis. They die faster, often within 7 days after onset of the disease. It is intriguing that rabies neutralizing antibody can be demonstrated in only 20% of humans with rabies (Hemachudha *et al.*, 1988). Few of them survived more than 12 days and yet no antibody could be demonstrated. This differs from cases previously reported where antibody to rabies virus usually develops if the patients survive more than 8 days after onset of disease (Anderson *et al.*, 1984). Hence, an insufficient antigenic mass in the early stage has been thought to be responsible for this phenomenon. However, the absence of detectable IgM class rabies antibodies even in the second week of illness in unvaccinated rabies patients with incubation periods of up to several months, as reported by Warrell *et al.* (1988) agrees with our findings, and suggests that intrinsic defects in immune recognition and/or activation process may also be underlying mechanisms.

Rabies virus contains five proteins. The nucleoprotein, non-structural protein and polymerase, designated N, NS and L, respectively, are linked to the viral RNA to form the helical structure. The glycoprotein (G) and matrix protein (M) are associated with the membrane. The glycoprotein forming the surface projections elicits both the production of a neutralizing antibody and of a cell-mediated immune response which, together, confer immunity against a lethal challenge in animals (Cox, Dietzschold & Schneider, 1977; Dietzschold *et al.*, 1982).

Recently, rabies virus nucleoprotein, which is more conserved antigenically among rabies virus strains than G protein, has been shown to protect mice or raccoons challenged peripherally with homologous or heterologous virus strains (Dietzschold *et al.*, 1987). The mechanism of protection could be induction of cytolytic T cells as well as T helper cells that augment the activity of virus-neutralizing antibody-producing B cells. Antibodies as well as rabies-specific T cell lines and clones directed against nucleocapsid protein have been demonstrated or isolated from recipients of inactivated rabies vaccine (Perrin *et al.*, 1986; Celis *et al.*, 1986). Antisera against antigenic domains of the N protein have been shown to destroy rabies-infected cells (Dietzschold, Rupprecht & Tollis, 1988). Antigen-

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Table 1. Characteristics of patients with rabies

Patient no.	Clinical type	Age (years)	Sex	Incubation period (months)	Days from onset to admission	Days from admission to death
1	Encephalitis	52	M	3	4	1
2	Encephalitis	19	M	1	3	1
3	Paralysis	63	M	1	7	2
4	Encephalitis	52	F	2	3	2
5	Encephalitis	34	F	1·5	4	1
6	Paralysis	43	F	3·5	8	1
7	Encephalitis	58	F	1	3	1
8	Encephalitis	33	F	12	3	1
9	Paralysis	48	M	2	9	2
10	Encephalitis	9	M	1	3	1
11	Paralysis	12	F	3	8	2

specific antibody to G or N possess the capacity to increase significantly the antigen-induced proliferative responses of human and murine rabies T cell lines and clones (Celis *et al.*, 1985).

In this study, serum levels of rabies neutralizing antibody and antibodies to N and G proteins were compared in rabies patients with those in rabies vaccinees. The purpose of this study was to determine whether there were differences in antibody responses to these antigens, particularly those directed against N protein.

PATIENTS AND METHODS

Patients

Eleven patients with rabies were admitted to Bumrasanaradura and Chulalongkorn Hospitals between January and December 1989. No history of rabies vaccination was obtained in any of these patients (Table 1). Nine healthy individuals who had received post-exposure prophylaxis for rabies using tissue culture rabies vaccine grown in Vero cells (Verorab; Institut Merieux, Lyon, France) were also included. The vaccine had been given intradermally according to the Thai Red Cross postexposure schedule: 0·1 ml intradermally at two sites on days 0, 3, 7 and at one site on days 30 and 90 (Chutivongse *et al.*, 1990). The diagnosis of rabies was made if there was a reliable history of dog or cat bite, typical clinical manifestations including aerophobia, hydrophobia and/or inspiratory spasms.

Rabies antibody estimations

Serum samples were taken on admission from patients with rabies and on days 0, 3, 7, 10, 14, 21, 28, 42 post-vaccination from rabies vaccinees. Patients with rabies were fully conscious at the time blood samples were collected, usually on the first day of admission (Table 1). The samples were stored at −70°C until assay. Rabies-neutralizing antibody in serum was estimated by the rapid immunofluorescent focus inhibition test (RIFIT) (Smith, Yager & Baer, 1973). The results were expressed in IU/ml. Antibody to N protein was measured in an ELISA. N protein was purified from rabies vaccine grown in chick embryo cells (Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) by SDS-gel electrophoresis. The purity was confirmed by comparing the preparation with standard

Table 2. Serological response to rabies G and N protein in human rabies

	Anti-G antibody		Anti-N antibody	
Patient no.	ELISA (OD)	RIFIT (IU/ml)	ELISA (OD)	IFA
1	0·252	Negative	0·151	< 1/5
2	0·333	Negative	0·257	< 1/5
3	0·261	Negative	0·240	< 1/5
4	0·150	Negative	0·137	< 1/5
5	0·445*	3·42	0·382*	1/20
6	0·135	Negative	0·170	< 1/5
7	0·249	Negative	0·158	< 1/5
8	0·237	Negative	0·240	< 1/5
9	0·274	Negative	0·241	< 1/5
10	0·580*	0·26	0·327*	< 1/5
11	0·640*	1·20	0·310*	< 1/5

*Considered as positive. Cut-off levels for assay of rabies G and N antibodies were an OD of 0·398 and 0·30, respectively.

RIFIT, rapid immunofluorescence focus inhibition test; IFA, indirect immunofluorescence antibody test.

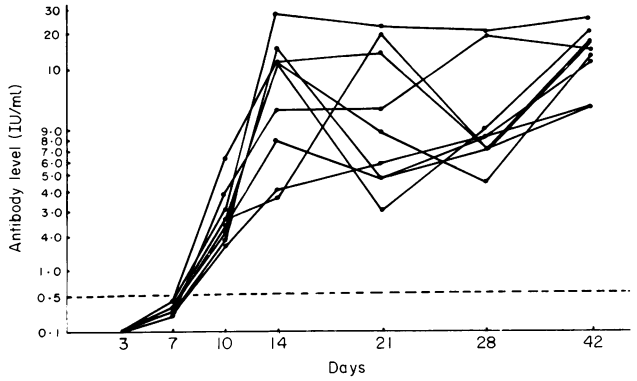

Fig. 1. Serum antibody levels to rabies virus in vaccine recipients as measured by RIFIT. — — —, protective level.

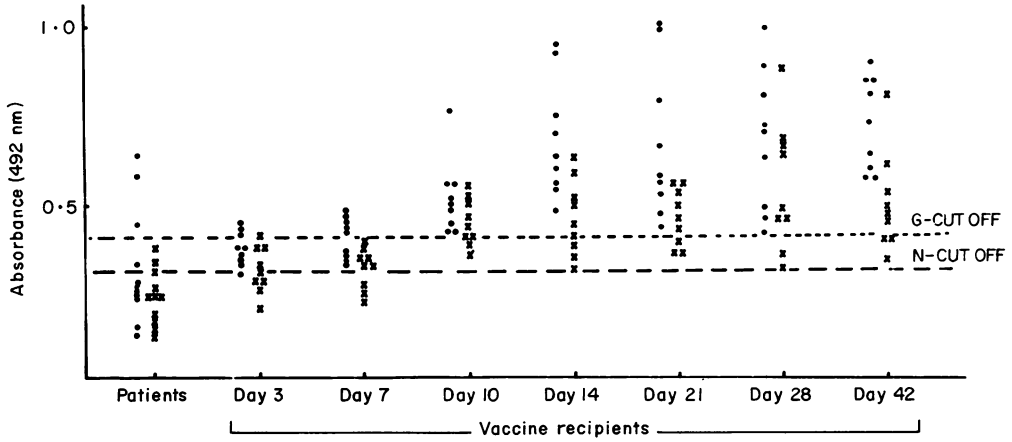

Fig. 2. Antibodies to G (●) and N (×) proteins of rabies virus were detectable by day 10 in all vaccinees by using ELISA. Three of 11 rabies patients had antibodies to both N and G proteins. Antibody level in ELISA was expressed as an actual OD. Cut-off levels for assay of G (- - -) and N (— — —) antibodies were 0·398 and 0·30, respectively (3 s.d. from the mean values for 11 control samples).

molecular weight markers and by immunoblotting using monoclonal antibodies to N and G proteins (gift of J. S. Smith CDC, Atlanta, GA, and M. Lafon, Institut Pasteur, France). Antibody to G protein was determined with an ELISA kit purchased from the Institut Pasteur, France. Antibody levels were expressed as the actual optical density (OD) value. OD values of at least 3 s.d. above mean of the day 0 sample in vaccinees were considered positive.

Presence of antibody to N protein in serum was confirmed by indirect immunofluorescence antibody test (IFA) (Larsh, 1965) using 95% acetone-fixed rabies-infected BHK cells and fluorescein-conjugated anti-human IgG, IgA and IgM prepared in goats (Kallestad, Chaska, MN) at a dilution of 1/20 in phosphate-buffered saline (PBS) as a secondary antibody. Non-immune human serum and fluorescein conjugated anti-rabies antibody prepared in horses (Becton Dickinson, Cockeysville, MD) served as controls. Titres were expressed as the highest serum dilution giving bright green, distinct, typical intracytoplasmic inclusions in the cells.

RESULTS

Clinical data

Of the 11 patients with rabies, seven had encephalitis and four had paralysis. The clinical details are shown in Table 1. The incubation period ranged from 1 month to 1 year in the encephalitic group, and was 1–3·5 months in the paralytic group. The time from onset of symptoms to death in all patients ranged from 4 to 11 days (Table 1). Survival times after onset of symptoms were shorter in patients with encephalitic rabies.

There were no complications in the vaccination group except a mild febrile illness after the first dose in two cases. These vaccinees were healthy after a follow-up period of 6 months.

Rabies-neutralizing antibody

Rabies-neutralizing antibody was detected in the serum of two rabies patients with encephalitis and in one with paralysis. Two patients (one encephalitic and one paralytic) had antibody levels above 0·5 IU/ml (1·20 and 3·42 IU/ml). The neutralizing antibody level in the other patient with encephalitis was 0·26 IU/ml (Table 2). None of the six patients, where CSF was available for assay, had demonstrable CSF rabies antibodies.

Rabies-neutralizing antibody with levels above 0·5 IU/ml was demonstrable in the serum of all rabies vaccinees on day 10 after initial vaccination. All of these vaccinees had detectable serum neutralizing antibodies to rabies on day 7. However, the levels on day 7 were below 0·5 IU/ml. Antibody levels were maintained throughout the whole study course of 42 days (Fig. 1).

Antibodies to rabies G and N proteins

Antibodies to rabies G and N proteins were detected in sera from three rabies patients. These were the same three patients who had neutralizing antibody in their sera. Cut-off levels for assay of rabies G and N antibodies in the ELISA test were an OD of 0·398 and 0·30, respectively. There was no correlation of the level of anti-rabies G and N antibodies and the time after onset of symptoms or with the length of the incubation period (Tables 1 and 2).

All of the healthy rabies vaccinees had antibodies to both G and N proteins detected on day 10 post-vaccination and later. Five and six vaccine recipients had an anti-N antibody response, whereas three and five vaccinees had antibody reactivity to G protein on days 3 and 7, respectively, as determined by ELISA (Fig. 2).

Results of IFA test for anti-N antibody were roughly in accordance with those of ELISA. However, this correlation was best at a high level of antibody as demonstrated in rabies vaccinees from day 10 and afterwards (Table 3, and Fig. 2). Serum titres were 1/20 or less in rabies patients, whereas titres of 1/20 or higher were observed in rabies vaccinees on day 10 post-vaccination (Table 3).

DISCUSSION

Antibody to rabies virus in the serum of unvaccinated rabies patients has been advocated as useful in the diagnosis of rabies in humans (Anderson *et al.*, 1984). Our recent experience, however, showed that only a minority of such patients (three out of 16) developed neutralizing antibody in the serum (Hema-

Table 3. Anti-N antibody (expressed as dilution titres) in vaccine recipients and rabies patients using immunofluorescence antibody test

Vaccinee no.	Days post-vaccination						
	3	7	10	14	21	28	42
1	< 1/5	< 1/5	1/80	> 1/320	> 1/320	1/80	1/40
2	< 1/5	< 1/5	1/80	1/80	1/40	1/40	1/40
3	< 1/5	< 1/5	1/80	1/80	1/160	1/160	1/160
4	< 1/5	< 1/5	1/20	1/40	1/40	1/80	1/160
5	< 1/5	< 1/5	1/40	1/40	1/80	1/160	1/160
6	< 1/5	< 1/5	1/20	1/40	1/40	1/80	1/80
7	< 1/5	< 1/5	1/40	1/80	> 1/320	> 1/320	> 1/320
8	< 1/5	< 1/5	1/80	1/80	1/80	1/80	> 1/320
9	< 1/5	< 1/5	1/20	1/20	1/20	1/20	1/40

Rabies patient no.	
5	1/20
10	< 1/5
11	< 1/5

chudha *et al.*, 1988). This study demonstrated similar results in that only one-fourth (three out of 11) patients with rabies had detectable rabies antibody. Such antibody, in three out of six patients of two series, was present as early as 3 days after onset of the first symptoms of rabies. In recipients of tissue culture rabies vaccine by the Thai Red Cross intradermal schedule, it was not until day 7 that rabies-neutralizing antibody can be detected. Such an early antibody response on day 7 after vaccination can also be seen in vaccinees receiving an intradermal as well as a 'four doses in 3 days' schedule of human diploid cell vaccine (Anderson *et al.*, 1981; Ratanavongsiri *et al.*, 1985; Warrell *et al.*, 1985). This suggests that in human rabies, the process of immune recognition and development of rabies antibody may occur earlier than the onset of clinical symptoms. Rabies antigen(s) may be 'seen' at the inoculation sites or at ganglion cells where initial viral replication occurs. Nevertheless, only a minority of cases develops serum rabies antibody before death.

In this study, rabies vaccine recipients who had anti-G antibody, also had a humoral response to N protein as determined by ELISA, RIFIT and IFA. Antibody levels to N and G proteins were roughly comparable in each recipient assayed at various times (Fig. 2). Titres of anti-N antibody in the vaccinees samples on day 3 or 7 were comparable with those of rabies patients on ELISA (Fig. 2, Table 2). This was also true using IFA. IFA titres of less than 1:5 were observed in two out of three ELISA anti-N antibody-positive rabies patients (Table 3). However, titres of anti-G as well as rabies-neutralizing antibodies in rabies patients were roughly at similar levels as those observed in vaccine recipients on day 10 (Figs 1 & 2, Table 2). Two out of three neutralizing-antibody-positive rabies patients had levels above 0·5 IU/ml (Table 2). These results suggest that rabies N component may be involved in order to have an adequate response to G protein. This had been previously demonstrated with influenza virus, where N protein of PR 8 strain can induce helper T cells that augment the function of B cells to produce virus-neutralizing antibody (Scherle & Gerhard, 1986). Similar results have been obtained using core protein of hepatitis B virus linked directly to a hepatitis B surface antigen (HBsAg) epitope (Milich *et al.*, 1988). Rabies N protein, by influencing cytolytic or helper T cells, offers protection from rabies in animals (Dietzschold *et al.*, 1987). Anti-N antibody, once developed, may in turn induce an antigen-induced proliferative responses of human and murine T cell lines and clones (Celis *et al.*, 1985). Further, monoclonal antibodies to internal proteins can block virus multiplication *in vitro* either by neutralizing newly translated N and NS proteins or by impairing the initial transcription of the genome (Lafon & Lafage, 1987).

It is still not known to what extent N protein plays a role in rabies pathogenesis, and which factors modulate immune responses to N and G proteins in human rabies.

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